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Proposal Category: Pest Management Strategies: Non-chemical strategies for pest control

Title: Evaluation of RNA-based biopesticide against *Botrytis cinerea*, the causative agent of Gray Mold of Brambles

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Abstract

Botrytis cinerea, the causal agent of gray mold, poses a major threat to global crop production, with the rising prevalence of fungicide-resistant strains raising concerns for human, animal, and environmental health. We have developed a mechanistically informed RNA-based biopesticide strategy that selectively silences essential fungal genes while minimizing off-target effects. Double-stranded RNAs (dsRNAs) were rationally designed to target key virulence regulators, cell wall biosynthesis enzymes, translation factors, and ergosterol pathway genes. Multi-gene targeting enabled pathway-level silencing, effectively inhibiting spore germination, germ tube elongation, and lesion development. Further, these constructs showed similar effect on fenhexamid-resistant isolates of *B. cinerea*. These results demonstrate that RNAi offers a highly specific, environmentally safe, and resistance-resilient alternative to conventional fungicides, establishing a scalable framework for sustainable crop protection that aligns with One Health principles and addresses the urgent need for innovative, safe disease management strategies

Introduction

Botrytis cinerea (*B. cinerea*), the causal agent of gray mold disease, is a pervasive and economically devastating pathogen affecting a wide range of crops worldwide, including fruits, vegetables, and ornamentals (Islam and Sherif, 2020). Its broad host range, high genetic variability, and capacity to infect under diverse environmental conditions make it a formidable challenge for crop protection efforts (Smagghe, 2025a). Conventional control relies heavily on synthetic fungicides, yet the repeated use of these chemicals has driven the emergence of fungicide-resistant *B. cinerea* strains with reduced sensitivity to multiple modes of action, compromising disease management and increasing environmental and health concerns (Rupp *et al.*, 2017; Islam and Sherif, 2020). These issues highlight an urgent need for innovative, sustainable, and safe disease management strategies that reduce reliance on chemical fungicides while maintaining effective control of gray mold.

RNA interference (RNAi) represents a promising alternative to traditional fungicides by enabling sequence-specific silencing of essential fungal genes via application of

double-stranded RNA (dsRNA) molecules (Islam and Sherif, 2020; Smagghe, 2025a). Exogenous application of dsRNAs designed against critical virulence factors has been shown to inhibit fungal growth and disease development on plants, including effective suppression of *B. cinerea* through spray-induced gene silencing (SIGS) (Wang *et al.*, 2016; Smagghe, 2025b). Targeting multiple genes simultaneously further enhances silencing efficacy and reduces the likelihood of resistance evolution compared with single-target approaches (Islam and Sherif, 2020). Recent studies have demonstrated that exogenous dsRNA can be internalized by fungal cells and trigger RNAi pathways, leading to significant reductions in lesion formation and pathogen virulence without detectable off-target effects on host plants or beneficial organisms (Wang *et al.*, 2016; Islam and Sherif, 2020). Such RNA-based biopesticides offer a highly specific, environmentally safe, and resistance-resilient alternative to conventional fungicides, establishing a scalable framework.

The study evaluated seven exogenously applied dsRNA constructs designed to target genes essential for *B. cinerea* virulence and development. After confirming effective gene suppression *in vitro* using RT–qPCR, the constructs were tested for their impact on spore germination and lesion formation both *in vitro* and *in planta*, including against fungicide-resistant isolates. Bioinformatic analyses indicated minimal off-target effects. Overall, these findings demonstrate that multi-gene RNAi represents an efficient, highly specific, and targeted strategy for managing gray mold disease.

Results

Target selection and construsts:

To evaluate the most promising RNAi targets in *B. cinerea*, seven genes were selected based on prior studies: the VELVET gene (*BcVEL1*), ergosterol 27 (BcERG27), chitin synthase 1 (*Bcchs1*), elongation factor 2 (*BcEF2*), and three ergosterol biosynthesis genes (*BcERG11*, *BcERG1*, and *BcERG13*) (Schumacher *et al.*, 2012; Nerva *et al.*, 2020; Duanis-Assaf *et al.*, 2022). Table 1 summarizes the function of each target gene, and Table 2 summarizes the constructs.

Table 1. Summarizes the target gene used in this study, its function and available fungicides.

Gene	Function	Fungicide Target?	Agricultural Fungicides / Class
velvet gene 1 (BcVEL1)	Global transcription factor controlling development, sporulation, secondary metabolism, and virulence	No	None (not a fungicide target)
chitin synthase 1 (BcCHS1)	Synthesizes chitin polymer for cell wall; essential for septa formation and hyphal integrity	Yes	Polyoxins (Polyoxin D, B); Nikkomycins (experimental)
EF2 (Elongation Factor 2)	Translation elongation factor; essential for protein synthesis	No (rare experimental inhibitors)	None in agriculture; mostly research
ERG13 (HMG-CoA synthase)	Catalyzes acetyl-CoA → HMG-CoA in the mevalonate pathway (early sterol biosynthesis)	No	None; research compounds exist (e.g., Hymeglusin)
ERG11 (C14-demethylase / lanosterol demethylase)	Catalyzes demethylation of lanosterol → key step in ergosterol biosynthesis	Yes	Azoles / DMIs: tebuconazole, propiconazole, difenoconazole, epoxiconazole, prothioconazole
ERG1 (Squalene epoxidase)	Converts squalene → 2,3-oxidosqualene in early sterol biosynthesis	Yes	Allylamines / Benzylamines (mainly medical: terbinafine; limited agricultural use)
ERG27 (3-keto sterol reductase)	Part of the C-4 demethylation complex, converts 3-ketosterols → ergosterol intermediates	No	None; affected only indirectly by morpholines

Table 2. Constructs in the present study.

Name	RNA type	Target
hpRNA^{FoVEL}	Inverted repeat	*Fusarium oxysporum* velvet gene (experimental control)
hpRNA^{BcVEL1}		BcVEL1
hpRNA$^{BcERG27+BcCHS1+BcEF2}$		*BcERG27+BcCHS1+BcEF2*
dsRNA$^{BcERG1+BcERG11+BcERG13}$	Double stranded	*BcERG1+BcERG11+BcERG13*
dsRNA$^{BcERG27+BcCHS1+BcEF2}$		*BcERG27+BcCHS1+BcEF2*
dsRNA^{BcVEL1}		BcVEL1
dsRNA^{BcCHS1}		*BcCHS1*
dsRNA^{BcEF2}		*BcEF2*
dsRNA^{BcERG1}		*BcERG1*
dsRNA^{BcERG11}		*BcERG11*
dsRNA^{BcERG13}		*BcERG13*
dsRNA^{BcERG27}		*BcERG27*

Effect of dsRNA on fungal spore germination:

To evaluate the effects of the RNA constructs on *B. cinerea* spore germination, spores were treated with dsRNAs targeting individual genes or defined gene combinations and examined microscopically 8 hours after incubation in 50% PDB. All dsRNA treatments reduced germination relative to controls (Figure 1), with the single-gene construct targeting *BcERGs* producing the strongest inhibition and *BcVEL1* dsRNA the weakest, suggesting that genes in the ergosterol biosynthesis pathway are more critical for early germination than regulators of later developmental transitions.

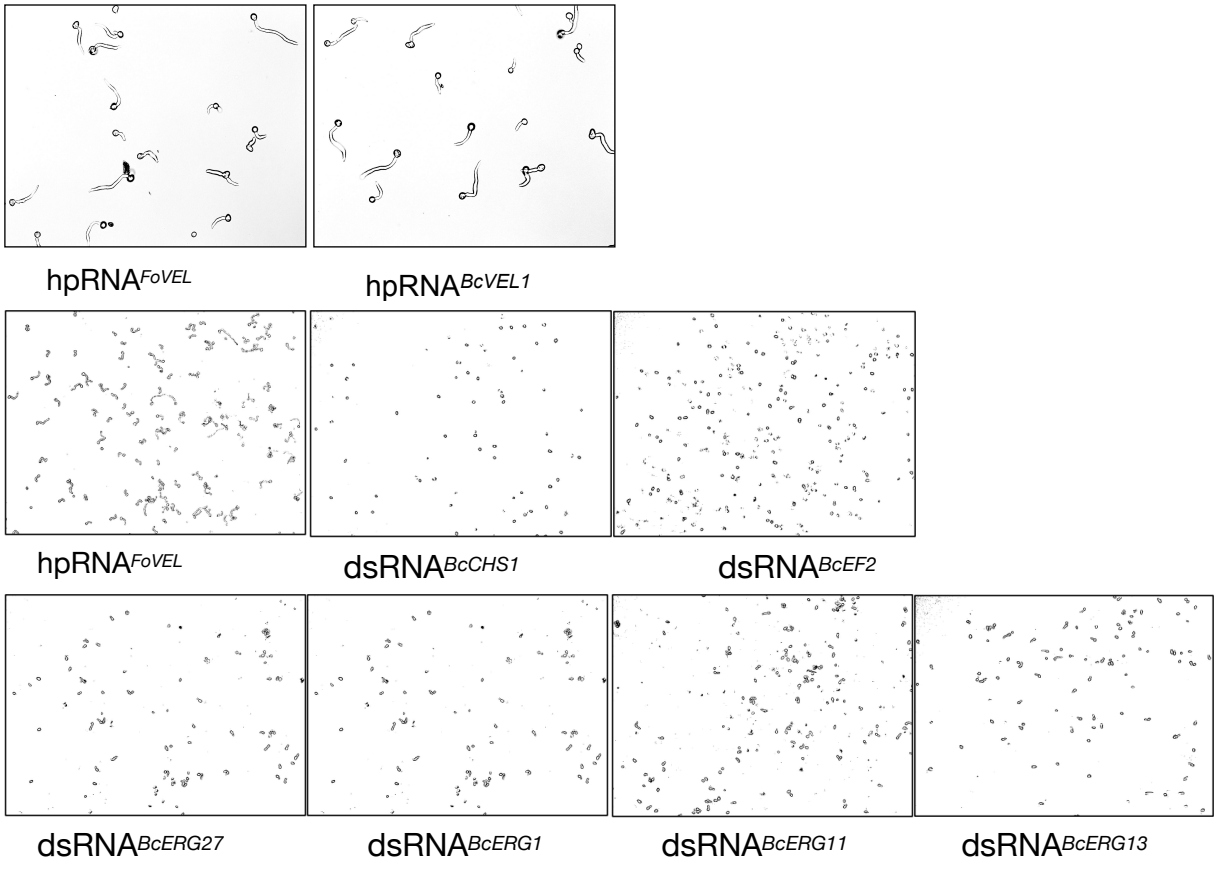

Figure 1. Effect of single-gene targeting on *B. cinerea* spore germination. Brightness and contrast of the original micrographs were adjusted to improve visualization.

Multi-gene treatments further amplified RNAi efficacy: the combination targeting *BcERG11*, *BcERG1*, and *BcERG13* achieved the most pronounced suppression, with only ~5% of spores germinating, while the few germinated spores

exhibited markedly shorter germ tubes compared with controls, indicating disruption of membrane biosynthesis and associated cellular expansion (Figure 2). These results demonstrate that simultaneous targeting of multiple *BcERGs* genes produces the strongest inhibition, outperforming single-gene and alternative multi-gene constructs, highlighting the mechanistic advantage of pathway-level RNAi in amplifying antifungal activity through coordinated silencing of functionally interconnected genes.

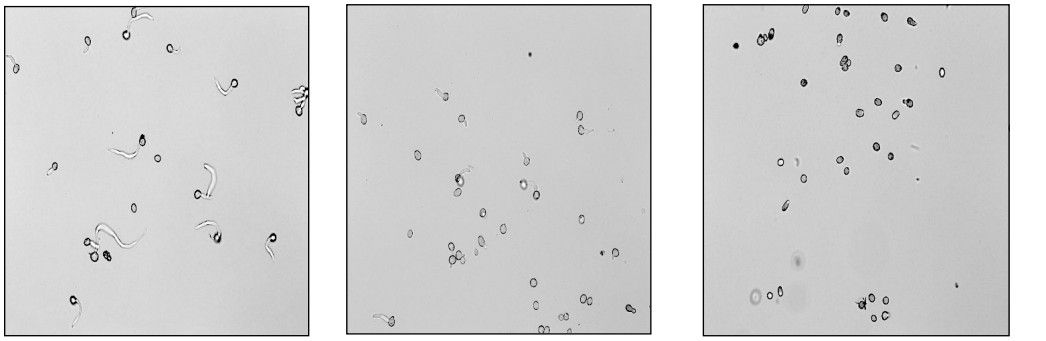

Figure 2. Effect of multiple gene targets on *B. cinerea* spore germination. Brightness and contrast of the original micrographs were adjusted to improve visualization.

Effects of RNA Construct Design on Spore Germination

dsRNA vs hpRNA**:** To investigate how RNA structure affects RNAi efficiency in *B. cinerea*, RNA was delivered either as purified dsRNA or as hairpin (inverted repeat) transcripts targeting *BcERG27+BcCHS1+BcEF2.*. For dsRNA, sense and antisense strands were synthesized separately and annealed by slow cooling in 200 mM Tris–Cl (pH 7.5) to form long, thermodynamically stable duplexes i.e. dsRNA$^{BcERG27+BcCHS1+BcEF2}$. These rigid helices serve as preferred substrates for fungal DICERs, which process them more efficiently than partially paired or flexible RNAs. Hairpin constructs were generated by *in vitro* transcription of inverted-repeat templates, producing intramolecularly folded dsRNA stems without requiring an annealing step (hpRNA$^{BcERG27+BcCHS1+BcEF2}$). As shown in Figure 3, the spores treated with both dsRNA$^{BcERG27+BcCHS1+BcEF2}$, and hpRNA$^{BcERG27+BcCHS1+BcEF2}$ showed effect on spore germination compared to control-treated *B. cinerea* spores (treated with hpRNA^{FoVEL}).

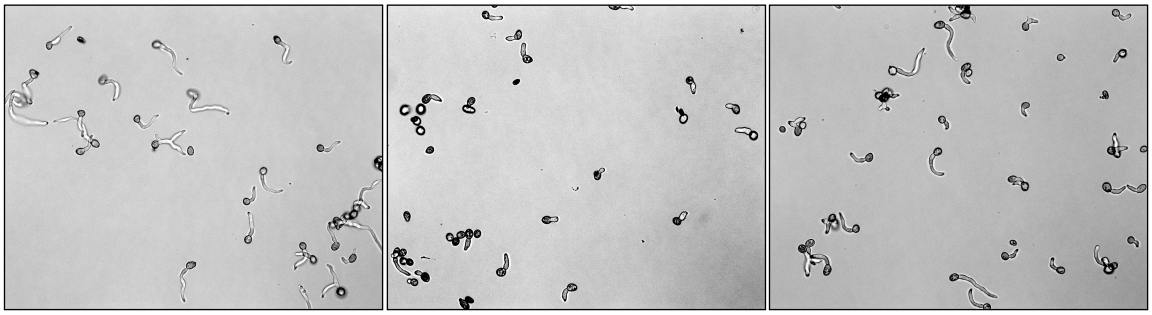

Figure 3. Effect of multiple gene targets on *B. cinerea* spore germination. In the figure, *B. cinerea* spores were treated with: (1) hpRNA^{FoVEL}; (2): dsRNA$^{BcERG27+BcCHS1+BcEF2}$, and (3) hpRNA$^{BcERG27+BcCHS1+BcE2F}$, respectively. Brightness and contrast of the original micrographs were adjusted to improve visualization.

Effect of RNA Construct on *B. cinerea* maturation

To study the effect of RNA silencing of different target genes on *B. cinerea,* the spores treated with RNA constructs were culture on PDA culture plates containing chloramphenicol. The PDA culture plates were incubated at room temperature for 7 days. As shown in Figure 4, the spores treated with hpRNA$^{BcERG27+BcCHS1+BcEF2}$ and dsRNA$^{BcERG1+BcERG11+BcERG13}$ whereas the spores treated with hpRNA^{BcVEL1} showed the least effect. These results agree with the spore germination assays.

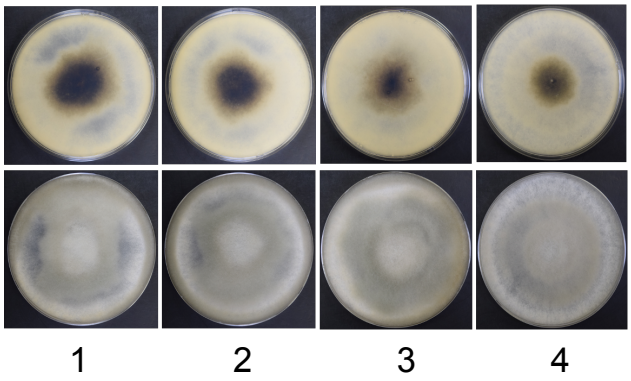

Figure 4. Effect of RNA constructs on *B. cinerea* development. In the figure, *B. cinerea* spores treated with: (1) hpRNA^{FoVEL}, (2) hpRNA^{BcVEL1}, (3) hpRNA$^{BcERG27+BcCHS1+BcEF2}$, and (4) dsRNA$^{BcERG1+BcERG11+BcERG13}$, respectively.

Multi-gene RNAi markedly reduces lesion development *in planta*

To assess the effect of dsRNA-mediated gene silencing on *Botrytis cinerea* lesion development, hpRNA^{BcVEL1}, hpRNA$^{BcERG27+BcCHS1+BcEF2}$, and dsRNA$^{BcERG1+BcERG11+BcERG13}$ constructs were evaluated on tomato leaves. Each RNA construct, prepared in sterile molecular-grade water, was mixed with *B. cinerea* spores and inoculated onto the leaf surface. At 5 days post-inoculation (dpi), all RNA treatments significantly reduced lesion development compared with the control (Figure 4). The strongest inhibition was observed with hpRNA$^{BcERG27+BcCHS1+BcEF2}$, *and* dsRNA$^{BcERG1+BcERG11+BcERG13}$, consistent with coordinated silencing of multiple genes involved in ergosterol biosynthesis. This enhanced suppression is likely mediated by DICER-dependent processing of long dsRNA into siRNAs targeting multiple components of the same metabolic pathway. In contrast, hpRNA^{BcVEL1} exhibited the weakest inhibitory effect. Collectively, these results demonstrate that multi-gene RNAi enables enhanced, pathway-level suppression of fungal virulence in planta.

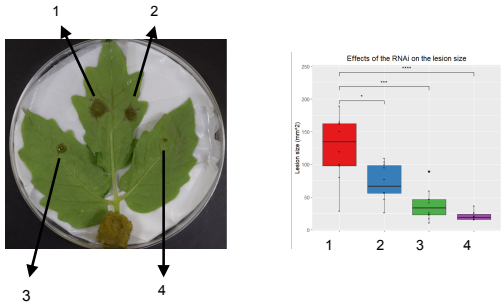

Figure 5. Effect of RNA constructs on *Botrytis cinerea* lesion development. In the figure, 1: *B. cinerea* spores inoculated with hpRNA^{FoVEL}, 2: *B. cinerea* spores inoculated with hpRNA^{BcVEL1}, 3: *B. cinerea* spores inoculated with hpRNA$^{BcERG27+BcCHS1+BcEF2}$, and 4: *B. cinerea* spores inoculated with dsRNA$^{BcERG1+BcERG11+BcERG13}$, respectively.

Future work plan

Collectively, the results obtained to date demonstrate that RNA silencing is an effective strategy for suppressing *Botrytis cinerea* growth, with particularly strong efficacy observed when simultaneously targeting multiple genes within the ergosterol biosynthesis pathway or across distinct functional pathways (BcERG27, BcCHS1, and BcEF2). Based

on these outcomes, we have identified “two lead RNA constructs” that are well positioned for advancement to field-level evaluation.

Translation of RNA-based disease control strategies to agricultural field conditions requires delivery systems that are scalable, cost-effective, environmentally safe, and compatible with existing crop management practices. While nanoparticle-based RNA carriers have shown promise, their field deployment is constrained by concerns related to environmental persistence, potential ecotoxicological effects, regulatory hurdles, production costs, and inconsistent RNA uptake by target pathogens as reported by us (Chen *et al.*, 2025; Adkar-Purushothama *et al.*, 2026). These limitations underscore the need for alternative delivery platforms that enable reliable, scalable, and sustainable field application. To address these challenges, future work will focus on the development of RNA delivery systems based on natural, biological, and biodegradable substances. Candidate carriers will include plant- and microbe-derived materials such as chitosan, cellulose- and lignin-based polymers, and protein- or polysaccharide-based biopolymers that are already widely used in agricultural formulations. These materials are compatible with conventional spray application methods and existing farm equipment, facilitating seamless integration into current crop management practices.

Importantly, biodegradable and biologically derived carriers offer rapid environmental breakdown, reduced persistence, and minimal off-target risk, thereby addressing key regulatory and ecotoxicological concerns associated with synthetic nanoparticles. This delivery strategy aligns with sustainable agriculture initiatives and regulatory frameworks, including PMRA and EU Green Deal priorities, and supports the development of environmentally responsible, RNA-based crop protection technologies suitable for field deployment and grower adoption. Successful implementation of this system would represent a significant step toward practical, field-deployable RNA-based crop protection technologies that meet regulatory requirements and sustainable agriculture goals. The outcomes of this work will be disseminated through publication in a peer-reviewed journal, anticipated in mid-2026.

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